

MOBILITY OF NORMAL AND VIRUS-TRANSFORMED CELLS IN CELLULAR AGGREGATES

HOWARD GERSHMAN and JUDITH DRUMM

From the Department of Biochemistry, Case Western Reserve University Medical School, Cleveland, Ohio 44106

ABSTRACT

The mobility of embryonic chick cells and cells of four established cell lines was examined in cellular aggregates. This was done by preparing aggregates of unlabeled cells and allowing cells of the same type, but prelabeled with [³H]thymidine, to adhere to the surface of the aggregates. After 2-½ days in agitated liquid culture the positions of the labeled cells within the aggregates were determined by autoradiographic techniques. Since the labeled and unlabeled cells were otherwise identical, the degree of penetration of the labeled cells into the aggregates was taken as a measure of the mixing or mobility of cells in the aggregate. With this procedure, embryonic chick liver, heart, and neural retina cells were found to move an average of 2.12, 2.68, and 4.00 cell diameters inward, respectively. Mouse fibroblast BALB/c 3T3 cells moved an average of 1.13 cell diameters inward, while Simian virus 40 (SV40)-transformed BALB/c 3T3 cells moved as much as 8.80 cell diameters inward, indicating that cells of the malignant SV40-transformed line were considerably more mobile than the corresponding nonmalignant 3T3 cells. In contrast, cells of the hamster fibroblast line NIL B moved 4.17 cell diameters in 2-½ days, while SV40-transformed NIL B cells moved 3.00 cell diameters in the same time. It was therefore concluded that infection with oncogenic viruses does not necessarily result in increased cellular mobility.

Observations on the behavior of cultured cells migrating on solid substrata such as glass or plastic have indicated that they can inhibit each other's mobility. The early work of Abercrombie and Heaysman (2, 3) and Abercrombie et al. (4) described this phenomenon and termed it "contact inhibition." Weston and Abercrombie (29) later predicted that in solid tissues this phenomenon would produce the effect of preventing random migration of cells and thus stabilize the tissue morphology. This prediction was in part confirmed by their experiments in which they fused together

two fragments of embryonic heart or liver in vitro and observed retention of the original fusion border, even after 2-½ days of culture.

On the other hand, more recent reports by Wiseman and Steinberg (30) and Armstrong and Armstrong (5) have suggested that cells in such solid masses are not totally immobile. Wiseman and Steinberg studied the same tissue types as Weston and Abercrombie: embryonic chick heart and liver, both as tissue fragments and cellular reaggregates. Instead of fusing two masses together, they allowed a few radiolabeled cells to

adhere to the surface of these masses and observed their position within the aggregate after a few days by autoradiographic techniques. Armstrong and Armstrong (5), on the other hand, employed the fusion technique of Weston and Abercrombie (29) with mesonephric mesenchyme from quail and chick embryos. In both of these more recent studies, in contrast to the earlier work, considerable cellular mobility was observed. The reasons for these apparent inconsistencies are not yet clear.

The original studies of "contact inhibition" of Abercrombie et al. were particularly interesting in that they pointed out differences between the mobility of malignant cells and that of nonmalignant cells (4). They observed the migration of two kinds of mouse sarcoma cells, chick heart fibroblasts and mouse skeletal muscle fibroblasts on solid substrata. They found that the nonmalignant heart and muscle fibroblasts displayed pronounced contact inhibition of colony outgrowth, with respect to both themselves and each other and also with respect to the sarcoma cells, while the sarcoma cells did not. Abercrombie et al. (4) suggested that such a failure of contact inhibition of movement *in vivo* permits invasiveness to occur.

This present study was initiated for two reasons. First, an effort was made to provide additional data that might be used to resolve the apparent discrepancies between the studies on chick embryonic tissue cells. While no clear answer has yet emerged, we have confirmed the mobility of chick heart and liver cells and expanded the studies to include embryonic neural retina cells, with similar results. The second purpose of the present study was to investigate the mobility of normal and malignant fibroblasts in cellular aggregates in order to extend Abercrombie's studies on normal and malignant cells to a culture system without artificial surfaces and therefore more representative of solid tissues. The substrata over which the cells in these present studies move are nonartificial, namely the surfaces of other cells. Because of this, the potentially artificial cell-dish adhesions that mediate mobility in monolayer culture are replaced by cell-to-cell adhesions. Carter (8, 9) and later Harris (21) presented evidence suggesting that the direction and speed of motion of cells on artificial substrata was inversely proportional to their adhesion to the surface. As a working hypothesis, we have considered that a similar relationship may be obtained in cellular aggregates and that mobility may be mediated by the strength of intercellular adhesion. The experi-

ments dealing with the adhesion of normal and transformed cells will be reported in a separate publication.¹

Furthermore, since the normal and malignant cells used by Abercrombie et al. (4) were considerably different from each other with respect to origin, the authors could not unequivocally attribute the differences they observed to the malignancy of the cells, as they themselves pointed out (reference 4, p. 276). In our work, comparable pairs of normal and malignant cell types were achieved by using two established cell lines and their Simian virus 40 (SV40)-transformed counterparts. The experimental design for the work reported here was essentially the same for all the cell types used and was modeled on that described by Wiseman and Steinberg (30).

MATERIALS AND METHODS

Chemicals and Radiochemicals

All chemicals were purchased from commercial sources and were either biological grade, if available, or else the highest grade available.

[*Methyl-³H]thymidine was purchased from New England Nuclear (Boston, Mass.) or Amersham/Searle Corp. (Arlington Heights, Ill.) and had a sp act of 18–20 Ci/mmol.*

Culture Media

Chick embryonic cells were cultured in Eagle's minimum essential medium in Earle's balanced salt solution, with addition of 10% horse serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. This medium is referred to as "MEM \times 1 plus horse serum." Cells of established lines were grown in MEM \times 1 plus horse serum and also in the following medium: Eagle's minimum essential medium with fourfold increased concentrations of vitamins and amino acids plus 10% fetal calf serum plus 100 U/ml penicillin and 100 μ g/ml streptomycin. This medium is referred to as "MEM \times 4 plus fetal calf serum." The results of the experiments were not affected by the medium used.

All media were purchased from Gibco (Grand Island Biological Co., Grand Island, N.Y.) either as the dry mix (MEM \times 4) or as liquid (MEM \times 1) and were stored at 4°C. Liquid media were used within 1 mo of purchase. Sera were purchased from the same source, stored at –20°C and used within 2 mo.

Chick Embryonic Cells

Chick cells were obtained from embryos of White Leghorn chickens at 6 or 7 days of incubation. Heart

¹ Gershman, H., J. Drumm, and L. Culp. Submitted for publication.

ventricles, livers, and neural retinas were removed by dissection and dissociated to single cells with trypsin as previously described (19). The only modification was the use of lower concentrations of trypsin (0.1%, [Difco Laboratories, Detroit, Mich.] 1:250).

Established Cell Lines

The four cell lines used in this study were obtained from stocks maintained by Dr. Lloyd Culp, Department of Microbiology, Case Western Reserve University, School of Medicine, Cleveland, Ohio. Mouse fibroblast BALB/c 3T3 and SV40-transformed BALB/c 3T3 (designated SVT-2) were originally derived from the A31 clone isolated by Aaronson and Todaro (1). Hamster fibroblast NIL B cells were originally isolated by Diamond (13). The SV-transformant of the NIL B line, designated SV-NIL, was produced by Dr. Culp as reported elsewhere (10). Each of these four lines was previously cloned in Dr. Culp's laboratory.

Cells were kept as frozen stocks and were used between the 12th and 18th passage. Lines were examined periodically for mycoplasma contamination, but none was detected. Cells were routinely grown in 32-oz glass bottles and subcultured using trypsin-(ethylenedinitro)l-tetracetic acid (EDTA) solutions (0.05% Difco trypsin 1:250 and 0.5 mM EDTA). Before use in an experiment, cells were plated out into 100-mm plastic culture dishes in 15-ml medium and grown as subconfluent cultures for 3–4 days (3–5 generations). The medium was changed 24 h after plating and every 48 h thereafter. Cells were removed from the dishes with ethylenedinitro)l-tetracetic acid (EGTA) (0.5 mM in Ca^{++} - Mg^{++} -free phosphate-buffered saline [Dulbecco's A solution] [PBS] and used as single cells or to make aggregates. Thus, cells used in these experiments were grown as subconfluent cultures (not more than 25% confluent) and were not harvested with trypsin. These cells reached the following densities if allowed to grow to saturation with daily medium changes: 3T3, 10×10^4 cells/cm²; SVT-2, 100×10^4 cells/cm²; NIL B, $15\text{--}20 \times 10^4$ cells/cm²; and SV-NIL, $> 200 \times 10^4$ cells/cm².

Formation of Aggregates

All glassware, instruments, and media used were either autoclaved or sterilized by filtration through nitrocellulose membranes. All procedures were carried out under sterile conditions at room temperature unless otherwise noted.

Chick embryonic cells or cultured cells were rinsed twice with medium (either MEM \times 1 plus horse serum or MEM \times 4 plus fetal calf serum). 3×10^6 cells were suspended in 5 ml of medium and centrifuged at 790 g for 5 min in a round-bottomed culture tube (13 \times 85 mm). The culture tube, with the pellet intact, was then incubated at 37°C in a gassed incubator (95% air, 5%

CO₂) for 1.5–4.5 h. While the pellets appeared solid after centrifugation, they were not coherent enough to be manipulated until after a period of incubation. Chick embryonic cells, which had been trypsinized, were non-adhesive for several hours and required 3–4.5 h to form a coherent pellet. Cultured cells, which had been removed from plastic dishes using EGTA, rapidly adhered to each other and formed a coherent pellet which could be manipulated after 1–2 h of incubation. The pellets were pried from the bottom of the culture tubes with a bent spatula, transferred to a 60-mm glass petri dish, and cut into cubical fragments 0.3–0.5 mm on a side using microscalpels.

The fragments were then transferred to the underside of a plastic petri dish lid, one fragment per 20- μ l drop. The petri dish lid was inverted over the dish bottom to form hanging drops and incubated at 37°C in a gassed incubator for 24 h. During this time the fragments rounded up into smooth-surfaced spheres which revealed no obvious crevices or cracks upon histological examination. After 24 h in hanging drop culture, the aggregates were collected for seeding with radioactive cells.

Labeling of Cells with [³H]Thymidine

Chick embryonic cells were labeled with [³H]thymidine as previously described (19), by adding two doses (20 μ Ci each) to the egg through a window cut in the shell. Doses were added 24 and 48 h before the cell suspensions were prepared.

Cultured cells were labeled by addition of [³H]thymidine to the medium at a concentration of 2.6 μ Ci/ml. Cells were grown in this medium for 2–3 days which was sufficient to label 98–100% of the cells. The amount of thymidine used here was found to have some inhibitory effects on the final yield of these cells, but in preliminary experiments this was found to be due to an inhibition of plating efficiency rather than of growth. To avoid this inhibition, cells were plated into 100-mm dishes in nonradioactive medium, and the [³H]thymidine was added 6 h later, after attachment of the cells. Under these conditions, the yields of radiolabeled and unlabeled cells were the same. The labeling conditions appeared to have no effect on the cell lines as judged by the following criteria: (a) histological appearance was normal; (b) repetition of selected experiments at low labeling levels (0.1 μ Ci/ml) produced the same mobilities as seen at high levels; (c) in control experiments, mixtures of radiolabeled and unlabeled 3T3 cells (or labeled and unlabeled SVT-2 cells) did not sort out (self-segregate)¹ indicating that cell-to-cell adhesiveness (26) had not been altered by damage or selection; (d) as noted above, yields of labeled and unlabeled cells were the same, indicating no significant selection for subpopulations and (e) when labeled and unlabeled 3T3 cells were replated onto dishes, nearly all cells of either type reattached within 6 h (93.1% and 97.4%, respectively) indicating that no significant percentage of the population was grossly damaged.

Seeding of Aggregates with Radioactive Cells

Aggregates were removed from hanging drops with a sterile pipette and transferred to a 10-ml flask in about 1 ml of medium. A portion of radioactive cells in suspension was added to bring the total volume to 2 ml. Usually 50–100 aggregates and about 10^6 radioactive cells were incubated together. The flask was left stationary at 37°C in a gassed incubator for 2 h, but every 15–30 min it was gently agitated to resuspend the single cells and disrupt any contacts between aggregates. The aggregates were then rinsed three times in fresh medium to remove unattached radioactive cells. In order to insure against fusion of aggregates and possible spurious internalization of surface cells, each aggregate was cultured in a separate container. The containers used were cylindrical 5-ml glass vials (1.3×4 cm) containing 1-ml complete medium and capped with a modified Bellco closure (Bellco Glass, Inc., Vineland, N. J.). The vials were agitated at 180–200 gyrations per min on a gassed gyrotory water bath shaker at 37°C.

Cell Viability

Chick embryonic cells have been routinely cultured as aggregates in a number of laboratories. Under the conditions employed here, the cells appear to be healthy and display no foci of necrosis. In addition, they maintain cytological and histotypic arrangements characteristic of the individual cell types. Heart cells and liver cells, for example, retain their periodic acid-Schiff-positive character throughout the culture periods employed here, indicating high levels of intercellular glycogen. Liver cells are observed to form occasional small tubules or vesicles lined with an epithelium after 3 or more days of culture. Fig. 1 *a* is a photograph of a portion of such an aggregate which has also been autoradiographed. Neural retina cells were observed to form rosettes of cell bodies surrounding bundles of axons particularly after 3 or more days of culture, although this was less common in the 2-½ day cultures studied here. A photograph of a portion of a neural retina aggregate is shown in Fig. 1 *b*. The central core of one of these rosettes is indicated by *R* on this photograph. In addition, heart, liver, and retina cells retained the ability to self-segregate or sort out when two cell types were intermixed. For these reasons, and also on the basis of general histological criteria, the chick embryonic cells appear to be healthy.

Cells of established lines seemed to be more sensitive to environmental alterations than the chick embryonic cells. They became necrotic within 1 day if the pH of the culture medium was allowed to go above 7.5 or below 7.2. Preliminary studies indicated that the SV40-transformed lines were more sensitive than the 3T3 or NIL B lines, particularly if cultured for longer than 4–5 days. To avoid this, aggregates were maintained for a maximum of 3-½ days in culture (1 day in hanging drops and an

additional 2-½ days in shaker culture). Preliminary experiments also indicated that these cells often became necrotic if they remained more than about 250 μ m (20–35 cell diameters, depending on the cell line) from the surface of the aggregate for extended periods of time (3 or more days). Care was therefore taken to limit the size of aggregates used to no more than 50 cells in diameter. These observations are in good agreement with studies on anoxia-induced necrosis of tumor cells grown in vivo (27) and in vitro (14). For example, cells in the center of an SVT-2 aggregate 50 cells in diameter (the largest used here) would be 158 μ m from the surface (see Tables II and III). Tomlinson and Gray (27) report a range of 160–200 μ m for the maximum thickness that a layer of nonvascularized tumor cells can achieve without necrosis. These authors also calculate that the maximum penetration of oxygen into a solid tumor is about 145 μ m, with a probable error in their calculations in excess of 30%. The diameters of the chick embryonic cell aggregates were sometimes larger than those of the established cell lines, but no necrosis was observed. In no case did any measured section exceed 200 μ m in radius.

Fig. 1 *c–e* are histological sections (not autoradiographed) of aggregates of 3T3 and SVT-2 cells. The mitotic index is low in these aggregates; however, the metaphase figures that are observed are distributed throughout all regions of the aggregates. Note especially the metaphase figures in Fig. 1 *d* and 1 *e*, indicated by arrows. Metaphase figures were far more common in 1-day-old aggregates than in those cultured 2 or 3 days. This (in conjunction with the data below) is interpreted to mean that cells are capable of completing a division initiated before aggregation, but have a much reduced capacity to initiate new divisions in aggregates.

Preliminary experiments indicate that cells maintained as aggregates are healthy although cell division is decreased or absent. The evidence supporting this contention is: (*a*) cells maintained in aggregates for 2–3 days have similar plating efficiencies as cells maintained as confluent monolayers for the same time.¹ All four of the lines used in this study were tested for plating efficiency by culture for 3 days as aggregates, trypsinization to obtain single cells, and replating into dishes. In each case the number of colonies formed on plastic dishes was similar to the numbers of colonies obtained from cells maintained as monolayers. (*b*) 80–100% of the cells originally put into aggregates could be recovered by trypsinization after 2 days.² Since the trypsinization procedure itself invariably destroys some cells, this suggests that aggregates contained about the same number of cells after 2 days as they did initially. (*c*) Aggregate diameters remained constant throughout the 3-½-day culture period, indicating no gross increase or decrease in total cell number, in confirmation of the trypsin recovery data above. (*d*) No foci of necrosis were observed, providing aggregate size was strictly controlled. (*e*) The rate of [³H]thymidine incorporation into cells maintained as aggregates for 3 days is one to two

orders of magnitude lower per cell than that of logarithmically growing cultures.²

While the lack of change in cell numbers could be due to rapid death and disintegration balanced by a rapid rate of cell division, the low rate of thymidine incorporation suggests that this is not the case. Apparently, cells maintained as aggregates are in a quiescent state. Their relatively high-plating efficiency indicates, however, that they are still viable.

Histology and Autoradiography

Aggregates were fixed in Bouin's fluid, dehydrated, and embedded in paraplast. Blocks were sectioned at 5 μm and sections affixed to slides. After deparaffinization and rehydration, the sections were treated with 5% trichloroacetic acid at 0°C for 10 min to remove unincorporated [³H]thymidine. The slides were rinsed and coated with Kodak NTB-2 photographic emulsion (Eastman Kodak Co., Rochester, N. Y.). After 2-5 wk of incubation in the dark at 4°C, the slides were developed, fixed, and stained through the emulsion with hematoxylin or hematoxylin and eosin.

Measurements of Histological Sections and Data Analysis

While it is obviously impossible to deduce the actual path taken by a randomly moving cell from a single observation, the following method was used to estimate the minimum distance a cell could have covered. The distance from the nearest edge of the aggregate section to the radiolabeled cell was measured using a Zeiss microscope equipped with $\times 40$ objective and $\times 10$ widefield eyepieces. One eyepiece contained an ocular micrometer previously calibrated against a stage micrometer. Measurements were made to the nearest division of the micrometer scale, which at $\times 40$ equaled 2.53 μm . Since measurements were made between the nuclear center, i.e., cluster of silver grains, and the nearest edge of the aggregate, a recently attached cell which had not flattened onto the surface of the aggregate would theoretically be recorded as a negative distance, that is, positioned outside the "edge." Instead, such unflattened cells were recorded as 0. The initial flattening of these cells was consequently not recorded as a distance moved. As a surface cell flattened even more, its nucleus could come to rest as much as 0.5 cell diameter beneath the surface. This would be recorded as a slight movement inward.

²Carrino, D., and H. Gershman. Personal communication. This has been carefully studied in the case of the BALB/c 3T3 line and its SV40 virus-transformant, SVT-2. Within 24 h of aggregation, the incorporation of [³H]thymidine into BALB/c 3T3 cells has decreased to one 100th that of logarithmically growing cultures. The incorporation into the SVT-2 cells drops to about one 10th the logarithmic rate. Incorporation remains at these low levels for at least 3 additional days.

Data were tabulated in micrometers and later converted to cell diameters. To determine the conversion factors, cells of each type were measured 0 and 2-1/2 days after seeding (1 and 3-1/2 days after the aggregates were formed), using a $\times 100$ oil immersion objective. These data are tabulated in the Appendix and Table III, along with the standard deviation (SD) for each measurement.

At the same time the position of a radiolabeled cell was determined, the diameter of the histological section in which it appeared was also measured. Although many sections were circular or nearly so, the averages of the major and minor axes were used if the sections were elongated. In order to minimize the possibility of including measurements from a tangential section of an aggregate, any section more than 20% smaller than the diameter of the modal-sized section was not included in the tabulations. The probability of a tangential section of a very large aggregate being included is therefore very small. In addition, any section more than 50 cells in diameter for the cultured cells or 60 cells in diameter for the chick cells (a rare event), was not tabulated in order to eliminate any possible bias as the result of the cell death in the center of a large aggregate (see cell viability, above). For each experimental point (i.e., each line in Table I or Table II), 50-100 aggregates were fixed, sectioned, autoradiographed, scored, and tabulated. The mean section diameter plus or minus the SD for each experiment also appears in Tables I and II.

The size and size variability of the aggregates were not measured before sectioning; however, minimum estimates of both can be made from the data in Tables I and II (column 4). A comparison of the section diameters in these tables indicates that within each experiment the range of diameters is fairly narrow (one SD equals 5-20% of the mean). This is to be expected, since the aggregates were originally cut manually from a large pellet and carefully selected for size similarity. In addition, as described above, the sections themselves were further selected. Because of this relatively narrow distribution, the aggregate diameters are roughly equal to the mean section diameters. Variation in aggregate size *between* experiments was somewhat larger, with aggregate section size ranging from 27.3 to 59.2 cells in diameter. Part of this variation is due to the difficulty in producing identical-sized aggregates in successive experiments, and part is due to different sizes of the various cell types used (see Table III). Since section sizes are expressed in cell diameters, identical-sized sections will contain more cells if the cells are smaller. For example, neural retina, SVT-2, NIL B, and SV-NIL, the four smallest cell types also have the largest section sizes (measured in cell diameters).

The size and size variability will have little effect on mean cell position when cells penetrate only a few cell layers, e.g., the zero time observations. Under these conditions, the aggregate is, in effect, infinite in size. On the other hand, when penetration is considerable, the influences of aggregate size and interexperimental varia-

bility become important. In the limiting case, when interpenetration of radiolabeled cells is complete, i.e., the radiolabeled cells are randomly distributed through the aggregate, the final mean position of the radiolabeled cells will be determined *entirely* by the aggregate size. For intermediate degrees of penetration, the influence of aggregate size and size variability will be more or less strongly felt. In order to compensate for size variability between experiments, and to estimate how closely toward complete interpenetration the radiolabeled cells have proceeded, a method of analysis has been devised which takes aggregate size into account. This method expresses the mean position of radiolabeled cells as a percentage of the mean position of a random distribution (complete interpenetration). The derivation and the testing of this model are described briefly in the section Results (see footnote 3) and in more detail in the Appendix.

The section thickness (5 μm) was chosen relative to nuclear size (about 3-4 μm) in order to minimize the possibility of a radioactive cell appearing in two successive sections (24). Preliminary experiments in which serial sections of selected aggregates were examined indicated that very few radiolabeled cells appeared in two successive sections.

RESULTS

Primary Chick Embryonic Cells

The mobility of 6-day-old embryonic liver cells was studied by seeding cells onto the surfaces of

³The limit of inward movement of a population of radiolabeled cells will be reached when they become randomly distributed through the unlabeled population. In order to assess how far toward this end point the liver cells had moved, the frequency distribution of cells randomly distributed in an aggregate section was calculated. This calculation is described in detail in the Appendix. In brief, a histological section of an aggregate is assumed to be a series of concentric circles, each circle one cell in radius larger than the next innermost circle. The number of cells in any given position is the circumference of that circle measured in cell diameters. The probability of finding a cell at any location is the circumference of its circle (in diameters) divided by the total number of cells in the entire section. With this model, a frequency versus distance histogram was constructed, and also the *average* position of a cell in a section was determined. Both of these are dependent only on the diameter of the histological section (provided all distances are measured in cell diameters). The calculated, average position of a cell in a section is therefore the expected mean position of a population of randomly distributed cells, and therefore the *maximum* distance from the edge of the section that can be reached by a population of initially nonrandomly distributed cells. This method of analysis serves two purposes. First, it provides some measure of how closely the cell movement

aggregates and following their movement inward. The results of these experiments appear in Table I, lines 1-4. When sections of aggregates fixed immediately after seeding were examined, the radiolabeled liver cells were located an average of 0.31 cell diameters inward. A rounded cell recently adhering to the smooth surface of the aggregate would be recorded as zero cell diameter inward (see Materials and Methods), while a flattened cell could be recorded as much as 0.5 cell diameter from the edge; therefore, the number 0.31 diameter probably reflects the varying degrees of flattening achieved during the 2-h seeding period. An autoradiograph of one of those sections, fixed immediately after seeding, is shown in Fig. 1 *a*. Two radiolabeled cells are visible on the surface of the aggregate.

After 2- $\frac{1}{2}$ days in culture the liver cells were located an average of 2.12 cell diameters inward (lines 3 and 4). The distances moved, 2.02 and 2.22 cell diameters, indicated good agreement between duplicate experiments. While this number suggests relatively little movement, it is somewhat misleading. A histogram of distance moved versus frequency is presented in Fig. 2, and shows that while 34% of the cells have not left the surface after 2- $\frac{1}{2}$ days, the remainder of the cells (66%) are distributed at varying distances from the surface, some in excess of 7 cell diameters inward. In fact, 15% of the cells measured were 4 or more cell diameters from the surface. In the case of liver cells, the mean positions³ that they could theoretically reach when randomly distributed were 5.25 and 5.78 diameters from the surface in the two experiments. The liver cells had therefore achieved 42% and 35% of these values after 2- $\frac{1}{2}$ days in culture.

The mobility of 6-day embryonic chick heart

has approached its end point (a random distribution). Secondly, it serves as a method of normalization. Aggregate size varies from experiment to experiment, both because of experimental variation and because the diameter of the cell types studied differs. Because of this, the results of parallel experiments cannot be directly compared. If the data are expressed as the ratio of observed position to the predicted position of a randomly distributed population, all results can be expressed on the same scale (0-100%). It should be noted, however, that the arithmetic mean position used here, either as distance moved in cell diameters, or in percent of random, is a somewhat misleading measure, since the cell positions are not normally distributed. The data in Tables I and II should therefore be used in conjunction with the histograms presented in Figs. 2-6 and 8-9.

ventricle cells was also studied and found to be quite similar to that of chick liver. The results of these experiments are given in Table I, lines 5–7, also in Fig. 3. Immediately after seeding, heart ventricle cells were located 0.34 cell diameters inward, again indicative of partially flattened cells. By 2-½ days after seeding, the radiolabeled cells were located an average of 2.68 cell diameters from the surface (mean of lines 6 and 7), or 56% of the mean position of a random distribution.

The histogram of the experiments with heart cells (Fig. 3) displays a distribution pattern very similar to that of liver. While 28% of the heart cells were still on the surface, 25% were four or more cell diameters inward.

Neural retina cells from 7-day-old chick embryos were also studied using this technique. In Table I, lines 8–11, show the results of these experiments. Immediately after seeding, retina cells were found to be an average of 0.24 cell diameter from the surface (mean of lines 8 and 9), which corresponds to 3% of maximum. After 2-½ days in culture the radiolabeled cells were located an average of 4.00 (lines 10 and 11) cell diameters inward, or 42% of random. A photograph of an aggregate fixed after 2-½ days is shown in Fig. 1 *b*. A number of radiolabeled cells are visible in this section and are indicated by arrows.

The frequency versus distance histogram for these experiments is shown in Fig. 4. Neural retina cells show a pattern similar to that of heart and liver, with 30% still on the surface after 2-½ days, but 29% of the cells are 4 or more cell diameters beneath the surface. Retina cells moved farthest in cell diameters, but when aggregate size is taken into account, they prove to be intermediate between liver (the least mobile) and heart (the most mobile) cells.

3T3/SV40-Transformed 3T3 Cells

BALB/c 3T3 cells were found to be less mobile than the embryonic cells studied here. Immediately after seeding, radiolabeled cells were found an average of 0.46 cell diameter from the surface (Table II, lines 1 and 2). By 2-½ days after seeding, they were located 1.13 cell diameters inward, on the average. This corresponds to 27% of the mean position of a random distribution in 2-½ days. The frequency distribution of these cells as a function of distance is shown in Fig. 5. Measured immediately after seeding, nearly all of the cells were still on the surface, with only a few cells (5%) 1 diameter inward, and none farther. By 2-½ days

after seeding, 98% of the cells were still within 3 cell diameters of the surface and only 2% were located 4 or more diameters inward.

In marked contrast to the 3T3 cells, cells of the SV40-transformed 3T3 line SVT-2 displayed an enhanced mobility. The results are shown in Table II, lines 4 and 5. Immediately after seeding, SVT-2 cells were found 0.89 cell diameter inward (line 4) corresponding to 14% of the mean position of cells randomly distributed. By 2-½ days after seeding, the average position of the radiolabeled cells was 8.8 cell diameters from the surface. This corresponds to 108% of the mean position of cells randomly distributed. (One of the autoradiographs of this experiment is presented in Fig. 1 *f*.) The frequency vs. distance histogram for this experiment is shown in Fig. 6 A. It can be seen from this figure that only 7.7% of the radiolabeled SVT-2 cells are still on the surface after 2-½ days. The observed frequencies of these cells from Fig. 6 A are also presented in Fig. 6 B along with the distribution expected if the SVT-2 cells were randomly distributed throughout the aggregate. The experimental data (open circles) show a good fit to the theoretical random distribution (solid line). The line determined by least squares (dashed line) is nearly identical to the random distribution line (solid). The least squares line shows a correlation coefficient of 0.99 with the calculated random distribution line, indicating that the difference between the two is negligible. This confirms the fact that the SVT-2 cells have become completely intermixed in 2-½ days.

Because the mixing of SVT-2 cells was so rapid, we examined the early stages of movement. The experiment was repeated and the mean position of the SVT-2 cells was measured at half-day intervals from 0 to 2 days. The results, shown on lines 6–10 in Table II and also in Fig. 7, indicate that SVT-2 cells move at a rate of 2.8 cell diameters per day and eventually reach 83–97% of the random distribution after 1-½–2 days in culture.

NIL B/SV40-Transformed NIL B Cells

Since cells of the virally transformed SVT-2 line moved much faster than cells of the corresponding nonmalignant 3T3 line, the mobilities of another transformed/nontransformed pair of lines were tested. These data are shown in Table II, lines 11–14 and Figs. 8 and 9. The contact-inhibited nonmalignant NIL B line was studied, and it was found that, immediately after seeding, NIL B cells were located an average of 0.47 cell diameter, or

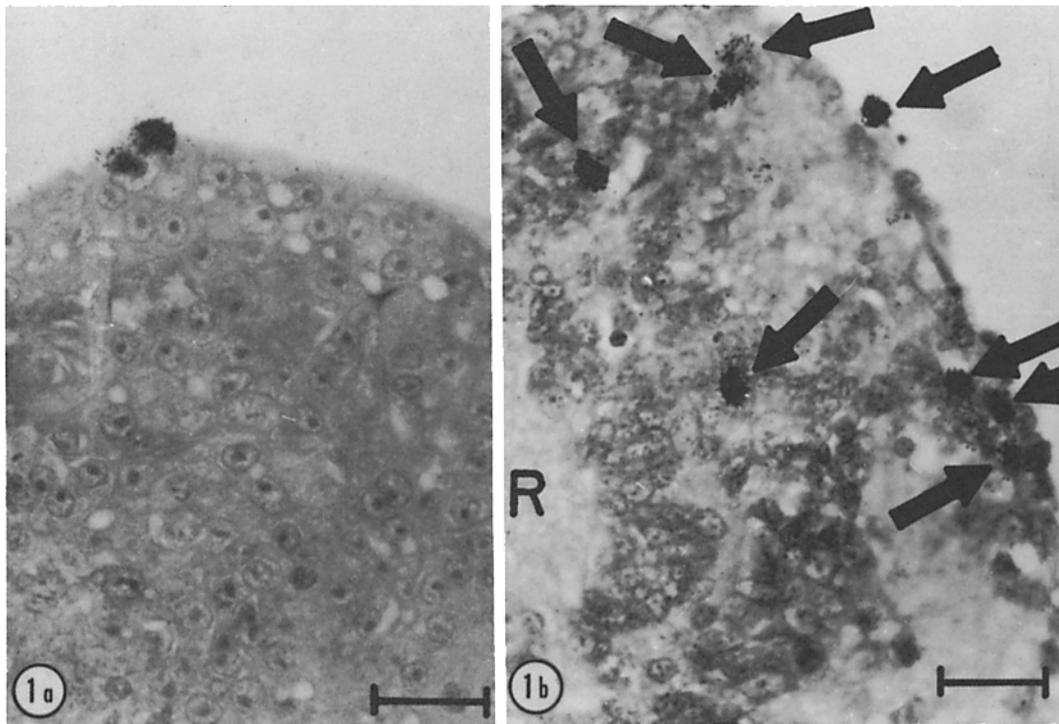


FIGURE 1 *a-f* (a) Autoradiograph of a histological section of an aggregate of 6-day-old embryonic chick liver cells. The aggregate was fixed immediately after incubation with a suspension of [^3H]thymidine-labeled liver cells. Two of the radiolabeled cells are visible at the surface of the aggregate. $\times 720$. Bar, $20\ \mu\text{m}$. (b) Autoradiograph of a histological section of an aggregate of 7-day-old embryonic chick neural retina cells. The aggregate was fixed 1- $\frac{1}{2}$ days after incubation with a suspension of [^3H]thymidine-labeled neural retina cells. Eight radiolabeled cells (indicated by arrows) can be seen at varying positions within the aggregate ranging from zero to eight cell diameters from the surface. Rosettes of retina cells consisting of cell bodies surrounding a central area packed with axons are also present. The core of one of these rosettes is labeled *R*. Axonal rosettes are not directly taken into account by the method of data analysis employed. To this extent the model oversimplifies the true geography of the aggregates. The axonal rosettes would be expected to introduce distortion of the model *only* if they constituted a large volume compared to the total cellular volume *and* if they were nonrandomly distributed. In this case neither of these occurs: the rosettes are fairly rare after 2- $\frac{1}{2}$ days of culture and are located randomly. There is even less of a deviation from ideality with heart, liver, and the established cell lines, which have no observable tendency to establish such oriented regions. $\times 670$. Bar, $20\ \mu\text{m}$. (c) Histological section of an aggregate of 3T3 cells cultured for 2- $\frac{1}{2}$ days as described in the text. This section was not autoradiographed. $\times 550$. Bar, $20\ \mu\text{m}$. (d) Histological section of aggregates of SVT-2 cells cultured for 2- $\frac{1}{2}$ days as described in the text. This section was not autoradiographed. $\times 225$. Bar, $50\ \mu\text{m}$. (e) Higher magnification of the aggregate in the center of Fig. 1 *d*. Note that the mitotic figures visible (some are indicated by arrows) are distributed throughout the aggregate. $\times 550$. Bar, $20\ \mu\text{m}$. (f) Autoradiograph of a histological section of an aggregate of SVT-2 cells. The aggregate was fixed 2- $\frac{1}{2}$ days after incubation with a suspension of [^3H]thymidine-labeled SVT-2 cells. Two of the radiolabeled cells (indicated by arrows) are visible deep within the aggregate. The focus in this micrograph is on the cells, and consequently the overlying silver grains are blurred. $\times 530$. Bar, $20\ \mu\text{m}$.

8% of random. By 2- $\frac{1}{2}$ days after seeding, the mean position of the NIL B cells was 4.17 cell diameters from the surface, or 77% of random. The NIL B cells, therefore, appeared to be considerably more mobile than cells of the other non-malignant line (3T3) studied. In fact, NIL B cells

proved to be more mobile than SV40-transformed NIL B cells. SV-NIL cells moved an average of 3.00 cell diameters (49% of random) in 2- $\frac{1}{2}$ days. In the case of the NIL B/SV-NIL pair, then, the malignant cells proved to be less mobile than the nonmalignant cells.

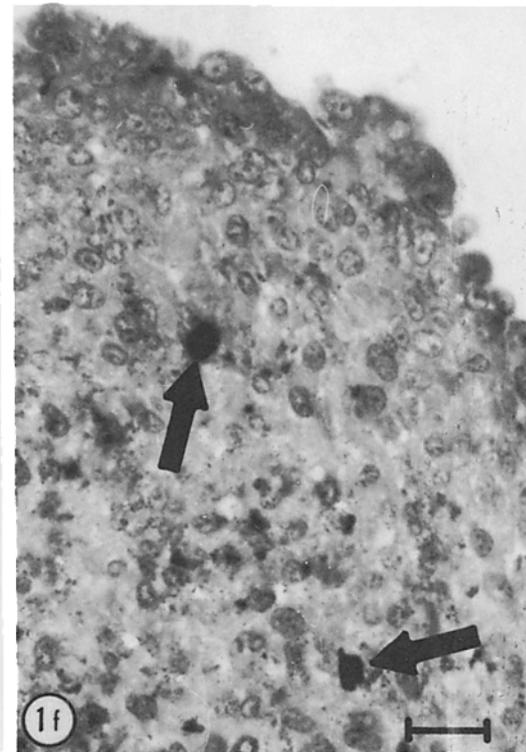
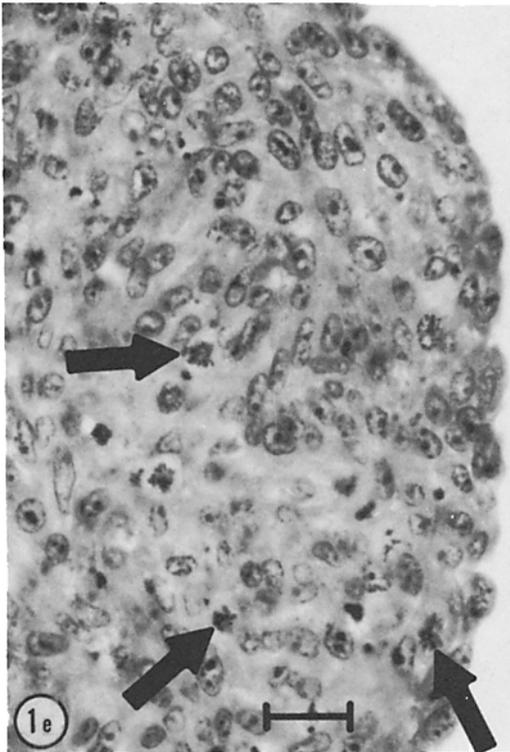
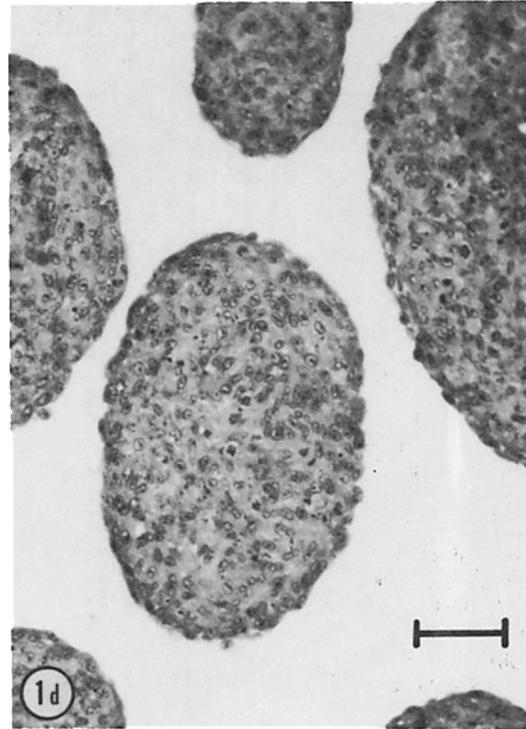
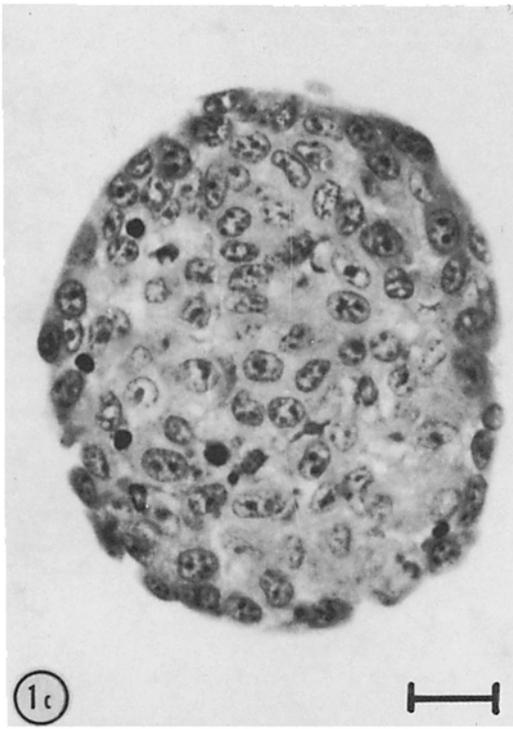


TABLE I
Movement of Chick Embryonic Cells into Homologous Aggregates

Line no.	Tissue	Days after seeding	Mean aggregate section diameter \pm SD*	No. of cells measured	Mean cell position		Ratio§
					Observed	Predicted†	
			<i>cell diameters</i>		<i>cell diameters</i>		%
1	Liver	0	29.6 \pm 3.8	338	0.31	4.64	7
2	Liver	0	30.4 \pm 3.8	342	0.31(0.31)	4.77	7 ⁽⁷⁾
3	Liver	2.5	33.3 \pm 5.4	502	2.22	5.25	42
4	Liver	2.5	36.5 \pm 7.1	850	2.02(2.12)	5.78	35 ⁽³⁸⁾
5	Heart	0	29.1 \pm 1.2	153	0.34	4.56	7
6	Heart	2.5	31.7 \pm 5.7	128	2.99	4.87	61
7	Heart	2.5	29.3 \pm 5.8	486	2.37(2.68)	4.59	52 ⁽⁵⁶⁾
8	Retina	0	39.1 \pm 5.8	914	0.31	6.21	5
9	Retina	0	47.0 \pm 7.2	541	0.18(0.24)	7.53	2 ⁽³⁾
10	Retina	2.5	56.5 \pm 12.6	1,129	3.22	9.10	35
11	Retina	2.5	59.2 \pm 9.8	939	4.79(4.00)	9.55	50 ⁽⁴²⁾

* *n* for these SD varied from 96 to 520. In general, they were 75–90% of the numbers in the column to the immediate right of the standard deviations.

† The calculation of the predicted theoretical mean position of cells in a random distribution is described in the Appendix.

§ Observed mean cell position/predicted mean cell position \times 100.

|| Numbers in parentheses are the means of the numbers to their immediate left.

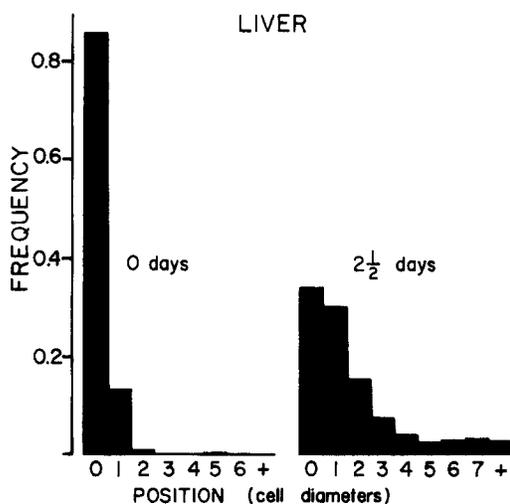


FIGURE 2 Distribution of radiolabeled embryonic chick liver cells within histological sections of liver aggregates immediately after (0 days) and 2-½ days after seeding. Ordinate, frequency with which cells were found (fraction of total cells observed). Abscissa, location of radiolabeled cells relative to the edge of the aggregate, in cell diameters; (+) stands for all locations greater than largest indicated location.

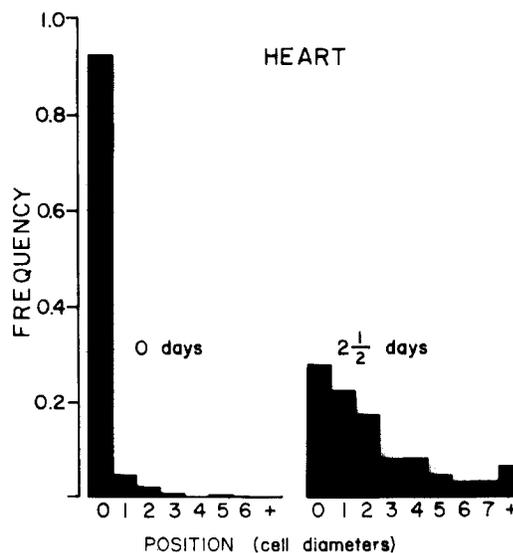


FIGURE 3 Distribution of radiolabeled embryonic chick heart cells within histological sections of heart aggregates. Other details are the same as Fig. 2.

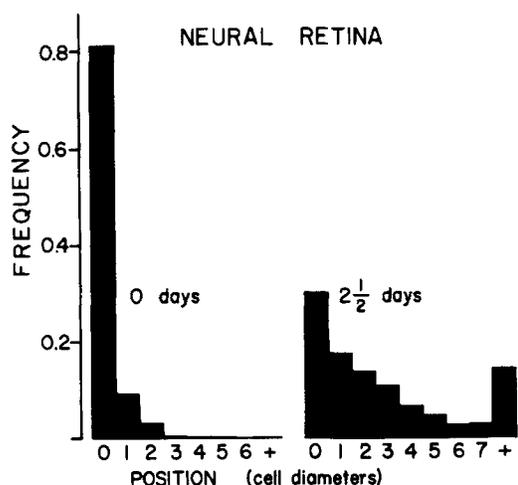


FIGURE 4 Distribution of radiolabeled embryonic chick neural retina cells within histological sections of neural retina aggregates. Other details are the same as for Fig. 2.

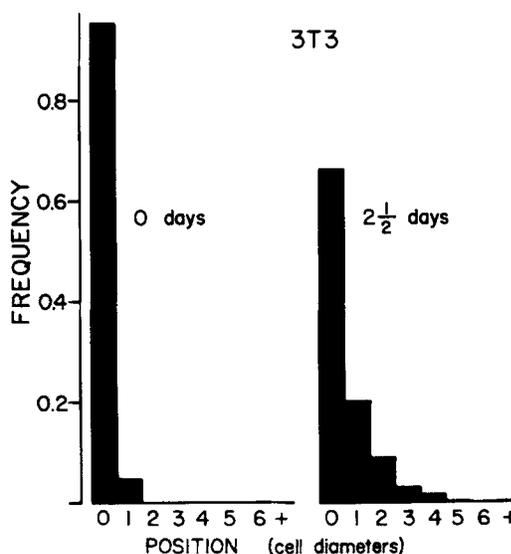


FIGURE 5 Distribution of radiolabeled BALB/c 3T3 cells in histological sections of 3T3 aggregates. Other details are the same as for Fig. 2.

TABLE II
Movement of Cells of Established Lines into Homologous Aggregates

Line no.	Cell line	Days after seeding	Mean aggregate section diameter \pm SD*	No. of cells measured	Mean cell position		Ratio§
					Observed	Predicted†	
				cell diameters		%	
1	3T3	0	28.3 \pm 4.2	184	0.38	4.42	9
2	3T3	0	29.2 \pm 5.8	208	0.55	4.57	12
3	3T3	2.5	27.3 \pm 3.1	635	1.13	4.26	27
4	SVT-2	0	39.3 \pm 3.6	597	0.89	6.25	14
5	SVT-2	2.5	50.6 \pm 5.6	430	8.80	8.12	108
6	SVT-2	0	32.5 \pm 4.0	244	0.49	5.12	9
7	SVT-2	0.5	36.3 \pm 2.4	227	1.77	5.75	31
8	SVT-2	1.0	39.4 \pm 3.4	167	3.33	6.26	53
9	SVT-2	1.5	39.3 \pm 3.4	71	6.05	6.25	97
10	SVT-2	2.0	38.9 \pm 4.3	138	5.14	6.18	83
11	NIL B	0	36.8 \pm 5.0	81	0.47	5.83	8
12	NIL B	2.5	34.2 \pm 5.1	197	4.17	5.40	77
13	SV-NIL	0	44.0 \pm 3.7	87	0.57	7.03	8
14	SV-NIL	2.5	38.7 \pm 7.0	184	3.00	6.15	49

* n for these SD varied from 56 to 409 aggregate sections measured. In general, they were 75–90% of the numbers in the column to the immediate right of the standard deviations.

† The calculations of the predicted theoretical mean position of cells in a random distribution is described in the Appendix.

§ Observed mean cell position/predicted mean cell position \times 100.

DISCUSSION

The data presented here confirm the observations of embryonic chick heart and liver cells cultured as of Wiseman and Steinberg (30) on the mobility of cellular reaggregates. In addition, we have ex-

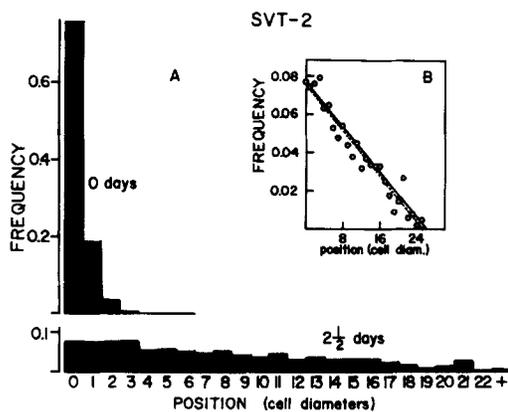


FIGURE 6 Distribution of radiolabeled SVT-2 cells in histological sections of SVT-2 aggregates. (A) Details are the same as for Fig. 2. (B) Composition of observed distribution of SVT-2 cells (open circles) with predicted frequencies, if cells were randomly distributed (solid line). This line was calculated as described in the Appendix, using an aggregate section diameter of 50.6 cells (Table II, line 5). The dashed line is the best fit to the observed points as determined by least squares. Ordinate, frequency with which cells are found; abscissa, location from edge of section (in cell diameters).

tended these experiments to a third cell type, embryonic chick neural retina. In all three tissue types studied here, the results were similar: considerable mobility of the radiolabeled cells was observed.

The apparent discrepancies between the work of Weston and Abercrombie (29), on the one hand, who found little movement of cells, and Armstrong and Armstrong (5) and Wiseman and Steinberg (30), on the other hand, who found considerable mixing, have not been resolved. Armstrong and Armstrong (5) suggested that differences in the tissue origin of the cells might explain the variance. However, since in the experiment of Wiseman and Steinberg (30), and also in the experiments reported here, the tissues used were the same as those used by Weston and Abercrombie (29) (embryonic chick heart and liver), this seems unlikely. Wiseman and Steinberg suggested that trypsin treatment of the radiolabeled cells might have stimulated their mobility. Armstrong and Armstrong, however, fused nontrypsinized chick and quail mesonephric mesenchyme fragments in their work and found considerable mobility. In addition, in the work presented here, cells of four established cultured lines were studied. The radiolabeled cells were removed from dishes with EGTA (which is believed to be less damaging to cells than trypsin

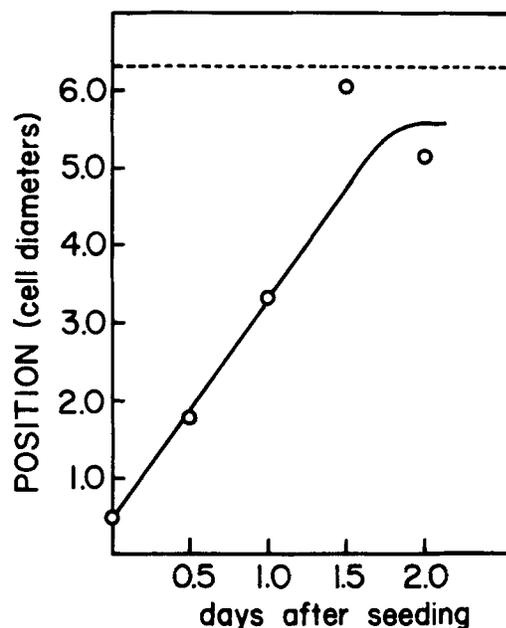


FIGURE 7 Position of radiolabeled SVT-2 cells in histological sections of SVT-2 aggregates as a function of time. Ordinate, mean position from edge of aggregate, in cell diameters; abscissa, number of days after seeding. The dashed line represents the mean position of cells if the distribution was random, i.e. the "maximum" movement possible. The value of this number is dependent on the size of the aggregate sections measured and therefore may vary for each value. In this figure, the line drawn has a value of 6.2 cell diameters. The individual values for the 0-, 0.5-, 1.0-, 1.5- and 2.0-day points were 5.0, 5.7, 6.2, 6.2, and 6.2, respectively. The section diameters from which these maximum values were calculated are given in Table II, lines 6-10.

[11]), and these cells were also found to be quite mobile in aggregates. This explanation, therefore, also seems unlikely to us.

A third possibility, not previously discussed, might account for these differences: the mobility of these cells may be affected by the serum used in the culture media. Specific factors that stimulate the migration of fibroblasts on solid substrata have been reported in a number of laboratories (6, 21, 31). These factors are present in different sera to varying degrees. Lipton et al., for example, report that the ability to stimulate migration of mouse 3T3 fibroblasts decreases in the order mouse > rat > human > fetal calf > calf > horse sera. Wiseman and Steinberg, Armstrong and Armstrong, and we employed Eagle's MEM with 10% horse serum as the culture medium. Weston and

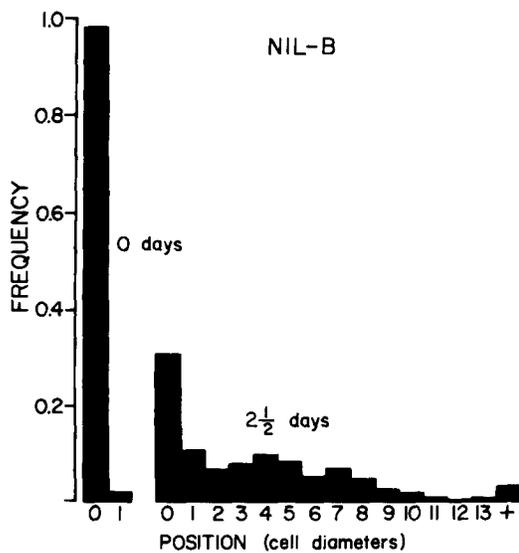


FIGURE 8 Distribution of radiolabeled NIL B cells in histological sections of NIL B aggregates. Other details are the same as for Fig. 2.

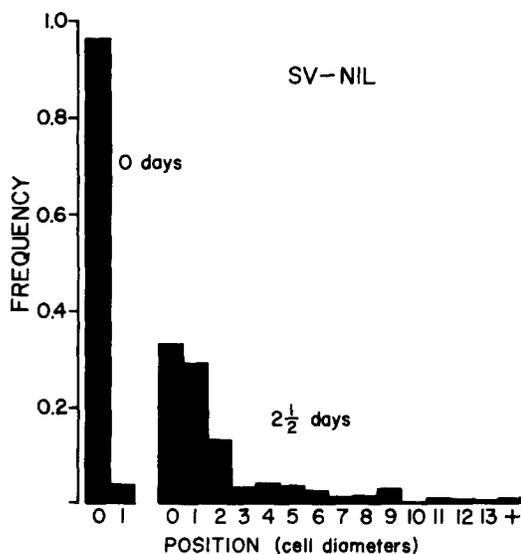


FIGURE 9 Distribution of radiolabeled SV-NIL cells in histological sections of SV-NIL aggregates; other details are the same as for Fig. 2.

Abercrombie, on the other hand, used a mixture of TC-199 and Hanks' salt solution with 20% chicken serum and 10% embryo extract. It is possible that a migration stimulating factor normally present in horse serum is absent from chicken serum or, alternately, that an inhibitor of chick cell migration is present in chicken serum or embryo extract. In our hands, no differences

between horse serum and fetal calf serum were observed with respect to the mobility of chick cells. This argues against this hypothesis, but, since we did not test chicken serum or embryo extract, the possibility cannot be eliminated.

The mobility of malignant versus nonmalignant cells and its relationship to invasiveness has been the subject of a number of studies (e.g., 4, 6, 16, 22, 28). For example, Abercrombie and his co-workers have shown that the advancing edge of a nonmalignant fibroblast culture is obstructed by other nonmalignant cells or by several kinds of sarcoma cells. The sarcoma cells, in contrast, are not obstructed by either the nonmalignant fibroblasts or other sarcoma cells (4). Abercrombie and his collaborators suggested that this process keeps normal tissues from mixing *in vivo* and that cells which fail to respond to this prohibition, such as cancerous cells, are invasive.

Gail and Boone (15, 16) have reported that while 3T3 and SV40-transformed 3T3 cells move at about the same rate in sparse cultures, the mobility of the 3T3 cells was inhibited in dense cultures, while that of the transformed line was not. Burk (6, 7) and Lipton et al. (21) have also shown differences in mobility of 3T3 and SV40-3T3 cells on solid substrata. The general conclusion from studies of normal and malignant cells has often been that malignant cells are more mobile than their nonmalignant counterparts (4, 7, 18, 25). It should be noted, however, that all of the above studies utilized fibroblasts moving on solid substrata. The movement of cells on such artificial substrata has been shown to be strongly influenced by the nature of the surface to which they are attached (8, 9, 12, 17, 20). The mobility of these cells on naturally occurring surfaces has not been investigated, and therefore the conclusions drawn from monolayer culture cannot be applied to *in vivo* situations without serious reservations. It therefore seemed important to us to examine the mobility of normal and transformed cells upon a more physiological substratum than glass or plastic, namely the surfaces of other cells. The movement of radiolabeled cells into the interior of cellular aggregates seem to us to provide a suitable experimental system for such studies.

The data presented in this report have shown that there is a striking difference in mobility of 3T3 and SVT-2 cells in cellular aggregates. The SVT-2 cells moved at a rate of about 2.8 cell diameters per day compared to a rate of movement for the 3T3 cells of 0.45 cell diameter per day (1.13

diameters in 2.5 days). This difference is very consistent with the observations of Gail and Boone for the same cells on solid substrata (16), and also with those of Martz and Steinberg for confluent cultures of 3T3 cells (23). It was therefore tempting, on the basis of these data alone, to conclude that viral transformation did indeed result in increased mobility. However, when a second pair of normal and transformed cell lines was examined, this relationship did not hold true. NIL B cells, a hamster fibroblast line, show moderate density-dependent inhibition of cell division. SV-NIL, the corresponding SV40 transformant of the NIL B, does not display density-dependent inhibition of cell division to any great degree and produces tumors in isogenic hamsters.* When the mobility of cells of these two lines was examined in cellular aggregates, SV-NIL cells did not display enhanced mobility, compared to NIL B cells. In fact, the difference observed, although small, indicates that SV-NIL cells may be *less* mobile than the nontransformed NIL B cells. This suggests that mobility, as measured by the assay employed here, is not necessarily increased in virally transformed lines relative to their nontransformed counterparts. It should be noted that the NIL B/SV-NIL pair of lines has not been characterized as extensively as the 3T3/SVT-2 pair with respect to tumorigenicity, invasiveness, or density-dependent inhibition of division. Because of this, extrapolation of the results beyond the simple observation that these two pairs of normal and SV40-transformed lines behave differently should be made with care.

It should also be noted that mobilities, as measured in aggregates, are a function of the movement of a given cell type over a specific substratum, namely the surfaces of cells of the same type. Invasiveness *in vivo*, on the other hand, is usually taken to mean the penetration of malignant cells that are moving not only over homologous malignant cell surfaces, but also over heterologous normal cell surfaces. The speed of movement of cells from a tumor invading normal tissues would presumably be influenced by three types of cell-to-cell interactions (normal-normal, normal-tumor, and tumor-tumor) as well as any intrinsic, nonadhesive mobility effects specific to the normal and tumor cell types not affected by cell-to-cell interactions. The movement of transformed or nontransformed cells through homologous aggregate

is therefore a considerably simplified model of the interactions that may occur *in vivo*. For this reason, speculations concerning the relationship between mobility in aggregates and invasiveness would seem to be premature.

APPENDIX

Calculation of Random Distributions of Cells in Histological Sections

The model given in Fig. 10 was used to calculate the theoretical random distribution of cells in an aggregate section: the section is assumed to be a circle, r_m cell diameters in radius, which consists of a series of concentric circles, each 1 cell diameter larger in radius than the next innermost circle. r_m = radius of the histological section in cell diameters and d_i = location of a cell in cell diameters from the edge of the section.

The total number of cells in this section, which are located d_i from the edge of the section, is the circumference of a circle (in cell diameters) of radius $r_m - d_i$, or:

$$C_i = 2\pi(r_m - d_i). \quad (1)$$

The total number of cells in the entire section is the sum of all such circles as d_i goes from 0 to r_m , or:

$$C_{\text{total}} = \sum_{i=0 \rightarrow r_m} C_i = \sum_{i=0 \rightarrow r_m} 2\pi(r_m - d_i) \quad (2)$$

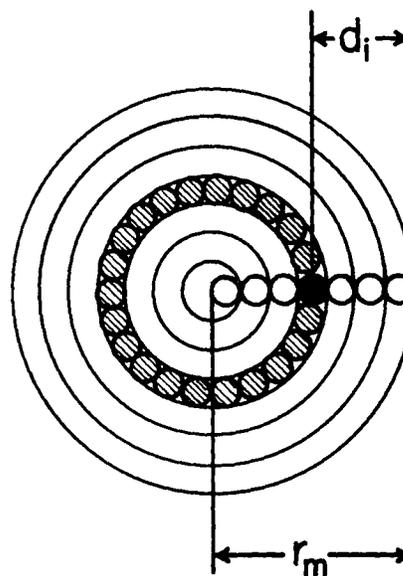


FIGURE 10 Schematic diagram of a histological section through an aggregate.

* Gershman, H. and R. Cook. Personal communication.

The probability, or frequency, of finding a cell at any given distance d_i from the edge is the ratio of C_i (see Eq. 1) to C_{total} (Eq. 2), or:

$$f_i = \frac{C_i}{C_{\text{total}}} = \frac{2\pi(r_m - d_i)}{\Sigma 2\pi(r_m - d_i)} = \frac{(r_m - d_i)}{\Sigma(r_m - d_i)} \quad (3)$$

The average location (\bar{d}_i) of a cell in such a histological section is the sum of the distances from the edge of the individual cells, divided by the total number of cells:

$$\bar{d}_i = \frac{\Sigma (d_i) (\text{no. of cells at distance } d_i)}{(\text{total no. of cells})} \quad (4)$$

$$= \frac{\Sigma (d_i) 2\pi(r_m - d_i)}{\Sigma 2\pi(r_m - d_i)} \quad (5)$$

and since:

$$\frac{2\pi(r_m - d_i)}{\Sigma 2\pi(r_m - d_i)} = f_i, \quad (6)$$

$$\bar{d}_i = \Sigma(d_i) (f_i).$$

The cell diameters actually used for these calculations are given in Table III.

Testing of the Theoretical Model

In order to test the validity of the assay, measurements were made of aggregates known to consist of a random mixture of labeled and unlabeled cells. To generate these aggregates, 3T3 cells were labeled with [^3H]thymidine according to the procedures in Materials and Methods and mixed with unlabeled 3T3 cells. Aggregates were

TABLE III
Diameters of Cells Cultured in Aggregates for 1 or 3-1/2 Days

Cell type	Cell size	
	μm	
Liver	10.00 \pm 0.91; 9.74 \pm 1.56*	
Heart	9.79 \pm 1.14; 10.16 \pm 0.71	
Retina	6.82 \pm 1.04; 6.67 \pm 0.63	
3T3	9.48 \pm 1.25; 8.96 \pm 1.35	
SVT-2	6.67 \pm 0.87; 6.25 \pm 0.98	
NIL B	6.11 \pm 0.75; 6.40 \pm 0.85	
SV-NIL	5.62 \pm 0.48; 5.51 \pm 0.50	

* First value \pm SD was measured in sections fixed immediately after seeding. n in each case was 10. Second value \pm SD was measured in sections fixed after 2-1/2 days in culture. In no case was there a significant difference between the two values.

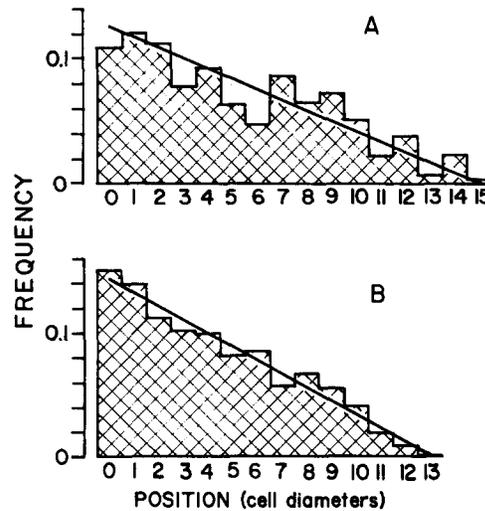


FIGURE 11 Distribution of radiolabeled cells in histological sections of aggregates consisting of random mixtures of labeled and unlabeled 3T3 (A) or SVT-2 (B) cells. Hatched histogram represents the observed distribution. Solid line is the predicted distribution. Other details are the same as in Fig. 2.

produced as described in the text, but not seeded, and cultured for 2-1/2 days to allow them to round up into smooth spheres. Histology and autoradiography were done as described in the text. Two histological sections of identical diameter (30 cells) were selected randomly, and the locations of all the labeled cells (a total of 256) relative to the surface were measured. This distribution is shown in Fig. 11 A. The theoretical random distribution was calculated using Eq. 3. These data are also shown in Fig. 11 A. The theoretical distribution, indicated by the solid line, shows a satisfactory fit with the observed data (correlation coefficient = 0.92; $\chi^2 = 0.0918$ [$P < 0.00001$]). The mean position (\bar{d}_i) was calculated from the cell positions, using the raw data and Eq. 4, and found to be 5.08 cell diameters inward from the surface. The average position for the theoretical line was calculated using Eq. 6, and found to be 4.93 cell diameters, in good agreement with the actual observations.

The same experiment, done with a mixture of labeled and unlabeled SVT-2 cells, is shown in Fig. 11 B. Three aggregate sections were measured, each 26 cells in diameter. As in Fig. 11 A, the theoretical line shows a good fit with the observed data (correlation coefficient = 0.98; $\chi^2 = 0.0131$ [$P < 0.00001$]). The mean location of labeled cells calculated from the observed positions was 4.13 cell diameters inward from the surface. The aver-

age location of cells in the theoretical distribution was 4.04. Again, the agreement was good. These data indicate that the theoretical method of calculating arithmetic means detailed above gives a close approximation of the true arithmetic mean, and that the theoretical predicted frequency distribution for a random distribution of cells closely approximates an actual random distribution of such cells.

The authors gratefully acknowledge the assistance of Dr. Lloyd Culp, who supplied the original cell lines and assisted in growing them.

This research was supported by American Cancer Society grant IN-57-L to Case Western Reserve University and by a grant from the Cuyahoga County Unit of the American Cancer Society to Howard Gershman.

Received for publication 22 November 1974, and in revised form 30 July 1975.

REFERENCES

1. AARONSON, S. A., and G. J. TODARO. 1968. Development of 3T3-like lines from BALB/c mouse embryo cultures: transformation susceptibility to SV40. *J. Cell Physiol.* **72**:141-148.
2. ABERCROMBIE, M., and J. E. M. HEAYSMAN. 1953. Observations on the social behavior of cells in tissue culture. Speed of movement of chick heart fibroblasts in relation to their mutual contacts. *Exp. Cell Res.* **5**:111-131.
3. ABERCROMBIE, M., and J. E. M. HEAYSMAN. 1954. Observations on the social behavior of cells in tissue culture. II. "Monolayering" of fibroblasts. *Exp. Cell Res.* **6**:293-306.
4. ABERCROMBIE, M., J. E. M. HEAYSMAN, and H. M. KARTHAUSER. 1957. Social behavior of cells in tissue culture. III. Mutual influence of sarcoma cells and fibroblasts. *Exp. Cell Res.* **13**:276-291.
5. ARMSTRONG, P. B., and M. T. ARMSTRONG. 1973. Are cells in solid tissues immobile? Mesonephric mesenchyme studied *in vitro*. *Dev. Biol.* **35**:187-209.
6. BURK, R. R. 1973. A factor from a transformed cell line that affects cell migration. *Proc. Natl. Acad. Sci. U. S. A.* **70**:369-372.
7. BURK, R. R. 1974. Some properties of a migration factor from a transformed cell line. *Cold Spring Harbor Conf. Cell Proliferation.* **1**:27-36.
8. CARTER, S. B. 1965. Principles of cell motility: the direction of cell movement and cancer invasion. *Nature (Lond.)*. **208**:1183-1187.
9. CARTER, S. B. 1967. Haptotaxis and the mechanism of cell motility. *Nature (Lond.)*. **213**:256-260.
10. CULP, L. 1974. Substrate-attached glycoproteins mediating adhesion of normal and virus-transformed mouse fibroblasts. *J. Cell Biol.* **63**:71-83.
11. CULP, L., and P. A. BLACK. 1972. Release of macromolecules from BALB/c mouse cell lines treated with chelating agents. *Biochemistry.* **11**:2161-2172.
12. CURTIS, A. S. G., and M. VARDE. 1964. Control of cell behavior: topological factors. *J. Natl. Cancer Inst.* **33**:15-26.
13. DIAMOND, L. 1967. Two spontaneously transformed cell lines derived from the same hamster embryo culture. *Int. J. Cancer.* **2**:143-152.
14. FOLKMAN, J., M. HOCHBERG, and D. KNIGHTON. 1974. Self-regulation of growth in three dimensions: the role of surface area limitation. *Cold Spring Harbor Conf. Cell Proliferation.* **1**:833-842.
15. GAIL, M. H., and C. W. BOONE. 1970. The locomotion of mouse fibroblasts in tissue culture. *Biophys. J.* **10**:980-993.
16. GAIL, M. H., and C. W. BOONE. 1971. Density inhibition of motility in 3T3 fibroblasts and their SV40 transformants. *Exp. Cell Res.* **64**:156-162.
17. GAIL, M. H., and C. W. BOONE. 1972. Time lapse studies on the motility of fibroblasts in tissue culture. *Ciba Found. Symp.* **14**:287-302.
18. GAIL, M. H., and C. W. BOONE. 1972. Cell-substrate adhesivity: A determinant of cell motility. *Exp. Cell Res.* **70**:33-40.
19. GERSHMAN, H. 1970. On the measurement of cell adhesiveness. *J. Exp. Zool.* **174**:391-406.
20. HARRIS, A. K. 1973. Behaviour of cultured cells on substrata of variable adhesiveness. *Exp. Cell Res.* **77**:285-297.
21. LIPTON, A., I. KLINGER, D. PAUL, and R. W. HOLLEY. 1971. Migration of mouse 3T3 fibroblasts in response to a serum factor. *Proc. Natl. Acad. Sci. U. S. A.* **68**:2799-2801.
22. MACIERIA-COELHO, A. 1967. Dissociation between inhibition of movement and inhibition of division in RSV transformed human fibroblasts. *Exp. Cell Res.* **47**:193-200.
23. MARTZ, E., and M. S. STEINBERG. 1974. Movement in a confluent 3T3 monolayer and the causes of contact inhibition of overlapping. *J. Cell Sci.* **15**:201-216.
24. MODAK, S. P., W. E. LEVER, A. M. THERWATH, and V. R. R. UPPULURI. 1973. Estimation of the proportion of cell nuclei in tissue sections falling within tritium-autoradiograph range. *Exp. Cell Res.* **76**:73-78.
25. RUBIN, H. 1966. Fact and theory about the cell surface in carcinogenesis. *Symp. Soc. Exp. Biol.* **25**:315-337.
26. STEINBERG, M. S. 1970. Does differential adhesion govern self-assembly processes in histogenesis? Equilibrium configurations and the emergence of a hierarchy among populations of embryonic cells. *J. Exp. Zool.* **173**:395-434.
27. TOMLINSON, R. H., and L. H. GRAY. 1955. The histological structure of some human lung cancers

- and the possible implications for radiotherapy. *Brit. J. Cancer* **9**:539-549.
28. VASILIEV, JU. M., and I. M. GELFAND. 1973. Interactions of normal and neoplast fibroblasts with the substratum. *Ciba Found. Symp.* **14**:311-329.
29. WESTON, J. A., and M. ABERCROMBIE. 1967. Cell mobility in fused homo- and heteronomic tissue fragments. *J. Exp. Zool.* **164**:317-324.
30. WISEMAN, L. L., and M. S. STEINBERG. 1973. The movement of single cells within solid tissue masses. *Exp. Cell Res.* **79**:468-471.
31. WOLF, L., and A. LIPTON. 1973. Studies on serum stimulation of mouse fibroblast migration. *Exp. Cell Res.* **80**:499-502.